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PRINCIPAL INVESTIGATOR: Dr. Joseph Quinn

CONTRACTING ORGANIZATION: Oregon Health & Science University
Portland, OR 97239-3098

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Dr. Joseph Quinn

Dr. Theresa A. Lusardi
Email: quinnj@ohsu.edu

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OREGON HEALTH & SCIENCE
UNIVERSITY 3181 SW SAM
JACKSON PARK RD PORTLAND OR
97201-3011

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14. ABSTRACT

shall state the purpose, scope, and major findings and be an up-to-date report of the progress in terms of results and significance. Abstracts will be submitted to the Defense Technical Information Center (DTIC) and shall not contain proprietary information. Subject terms are keywords that may have been previously assigned to the proposal abstract or are keywords that may be significant to the research.

A history of TBI increases the odds of developing AD by 2.5 times in the general population, and 4-6 times for military veterans. Although significant associations between mTBI and risk of AD have been observed, the precise mechanism by which TBI might lead to AD and/or AD-related symptoms are not yet understood. Histologically, AD is characterized by amyloid- and neurofibrillary protein aggregates, suggesting a loss of protein processing is a key feature of AD. MiRNAs are small non- coding RNA that regulate mRNA transcription, and may be a significant cause of protein dysregulation.
To date, we have established molecular biology techniques that allow us to measure miRNA in CSF from living donors. We have established and validated a biostatistical pipeline to identify biomarker candidates from our assay. We are building a bioinformatics pipeline to associate altered miRNA signatures with predicted changes in mRNA regulation, that may link altered miRNA with AD-related pathologies.

15. SUBJECT TERMS

Nothing listed

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1. Introduction

A history of TBI increases the odds of developing AD by 2.5 times in the general population, and 4-6 times for military veterans, and accelerates cognitive decline. Although significant associations have been observed, the precise mechanism by which TBI might lead to AD and/or AD-related symptoms are not yet understood. Protein biomarkers related to known AD pathologies and measured in CSF are very sensitive markers of AD, but they lack specificity, often showing up in individuals with no clinical signs of AD. It is not clear whether these protein biomarkers reflect necessary, but insufficient, processes leading to AD, or whether they reflect an early disease stage that, given enough time, will lead to AD. Histologically, AD is characterized by amyloid- and neurofibrillary protein aggregates, suggesting a loss of protein processing is a key feature of AD. MiRNAs are a recently discovered class of small non- coding RNA that regulate mRNA transcription, and may be a significant cause of protein dysregulation. Our investigative team has generated preliminary data showing that the miRNA distribution in CSF is altered in civilians with Alzheimer's disease (AD). Specifically, the signature of miRNA expression in AD is a decrease in abundance or the absence of a subset of miRNAs, which is consistent with the signature pathology of protein over expression and accumulation in AD. Further, when considering the TBI history of our subjects, we find that those with a history of TBI are over-represented in our AD group, and we find a specific group of miRNAs regulated in this population. We hypothesize that TBI induces an alteration in CSF miRNA patterns that reflect the initial molecular responses to brain injury that precede, and likely drive, changes in protein expression that lead to the development of AD. We have additional preliminary data showing altered protein biomarkers in deployed veterans with a history of TBI, and additional protein biomarkers specific for deployment, regardless of TBI history.

2. Keywords

Mild traumatic brain injury (mTBI), Alzheimer's disease (AD), miRNA, cerebral spinal fluid (CSF), biomarker, deployment, blast injury

3. Accomplishments

What were the major goals of the project?

Specific Aim 1: CSF miRNA measurement from mTBI and Controls

Major Task 1: Regulatory Approval *Complete 7/2016*

Major Task 2: CSF miRNA Measurement from mTBI and Controls *20% Complete*

Major Task 3: Identify Candidate Biomarkers *50% Complete*

Specific Aim 2: Identify miRNA as Biomarkers of mTBI

Major Task 4: FITBIR Data Sharing *20% Complete*

Major Task 5: Biostatistical Modeling

Major Task 6: Biomarker Candidate Verification

Specific Aim 3: AD Pathway-Directed Bioinformatics Evaluation of mTBI-regulated miRNA

Major Task 7: Bioinformatics Analyses *20% Complete*

Major Task 8: Manuscript Preparation

What was accomplished under these goals?

EWOF: Due to regulatory delays, an extension without additional funds has been granted to extend the period of performance for this project to 7/31/2018 (1 year extension, Amendment P00002). No other changes in the project scope or outcomes has been made.

Major Task 2: Standardized CSF miRNA qPCR, analysis, and quality control protocols have been established. Experimental samples have been received from UW (Table 1). A reference standard pool of CSF has been collected and stored according to the same protocol used to obtain the experimental samples. RNA isolation is ongoing, with one reference standard sample processed for each batch of 8 samples (7 experimental + 1 reference standard). The results from the reference standards will be used in Task 3 to correct for batch processing effects.

Table 1: Recruitment

Donor Group	***Target Enrollment***	***Current Enrollment***	***Age***
Veterans, with mTBI	50	47	33 +/- 9.9
Veterans, no mTBI	50	18	32 +/- 7.1
Civilians, no mTBI	60	53	34 +/- 9.3

Major Task 3: We have developed a statistical pipeline to process and identify candidate miRNA biomarkers measured using TaqMan Low-Density Arrays (TLDA). Individual arrays are processed using QuantStudio software (LifeTechnologies). Amplifications are reviewed and quantified using ExpressionSuite software (LifeTechnologies), which returns a Ct quantification value along with an amplification score indicating the quality of individual amplifications, and a confidence score, which indicates the goodness of fit for the Ct threshold value for a given miRNA target. Based on these flags and detection threshold (typically 35 Ct), each amplification value is flagged as "censored," indicating that it is below the reliable detection threshold; "excluded," indicating a poor amplification curve that cannot be reliably interpreted; or "good" indicating an acceptable amplification and quantification process. These results are then filtered according to their group performance and detection rates – targets that are not consistently represented within a group are excluded. This step limits the multiple corrections penalty in large-dimension studies by excluding targets that have poor representation and are thus less likely to provide reliable information to a statistical model. Results are then processed using four statistical models – linear regression and receiver-operator curves, linear methods that evaluate performance on an individual target basis, and CHAID (chi-square automatic interaction detector) and CART (classification and regression trees), classification and regression methods that evaluate individual performance in a group. MiRNA targets are considered candidates for further study if they are significant in at least 3 of the 4 methods.

In a closely related study comparing miRNA in CSF from individuals with Alzheimer's Disease (AD) to age matched, cognitively normal donors (Control) from the Oregon Alzheimer's Disease

Center (OADC) bank, we identified 36 candidate miRNA that distinguish AD from Control using this method. We have evaluated these 36 candidates using a custom TLDA array, using CSF from the Shiley-Marcos Alzheimer's Disease Research Center (SM-ADRC) in San Diego, and found that 26 of these candidates perform well in samples from a second bank.

The methods used for CSF collection and storage, and miRNA assay are identical to those that are underway for this project, and thus we expect that these statistical methods will be similarly applicable for the current project.

Major Task 4: We have established FITBIR pseudoGUIDs for all of the subjects in the project. Conversations are ongoing among FITBIR, UW/VA Puget Sound, and OHSU to arrange additional reporting as permitted by the originating IRB.

Major Task 7: Development of the bioinformatics pipeline is ongoing. The first step in the pipeline is to integrate results from multiple miRNA target prediction programs into a single set of predicted targets for a group of miRNA. We use predictions from TargetScan (Agarwal, Bell et al. 2015), rna22 (Miranda, Huynh et al. 2006), mirSVR (Betel, Koppal et al. 2010), and PITA (Kertesz, Iovino et al. 2007) to form a consensus prediction, which has been shown to maximize sensitivity while minimizing false positives (Oliveira, Bovolenta et al. 2017). Integrating results across these four prediction algorithms requires converting annotations to the same database (Ensembl, release 89), and ranking genes within each algorithm to identify the most likely regulated target mRNA. Genes will be included in the pathway analysis step if they meet the cutoff criteria in at least two of the target prediction algorithms.

Tasks 5, 6: Due to delays, these tasks will be completed in the next year. Despite the delays, we do not anticipate any technical difficulties or budget impacts in completing these tasks.

What opportunities for training and professional development has the project provided?

Nothing to Report.

How were the results disseminated to communities of interest?

Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?

During the next period, we will complete the CSF miRNA assays (Task 2), and complete data reporting to FITBIR (Task 4). We will use our statistical pipeline to assess miRNA differentially regulated between Veterans with mTBI and community controls (Task 3). We will use a correlation analysis to understand whether early mTBI-associated miRNA dysregulation is similar to those in established Alzheimer's Disease, or a potential precipitating event. We will use our bioinformatics pipeline to predict mRNA regulated by altered miRNA, and perform pathway analyses to test for enrichment in known AD related pathways (Task 7). Based on the correlation to miRNA altered in AD, we will perform additional pathway analyses to predict mechanisms that may link mTBI-related changes in miRNA to the development of AD-related pathologies. Biostatistical modeling will be utilized to assess performance of groups of candidate biomarkers, alone and in combination with other measures (ApoE status, A-beta, tau, etc) to identify blast exposure and potential risk subgroups (Task 5). Additional analyses will assess the influence of deployment on candidate miRNA. Predicted significantly regulated miRNA will be verified (Task 6), and a manuscript prepared (Task 8).

4. Impact

What was the impact on the development of the principal discipline(s) of the project?

Nothing to Report.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

5. Changes/Problems

Changes in approach and reasons for change

Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them

Due to regulatory delays, an extension without additional funds has been granted to extend the period of performance for this project to 7/31/2018 (1 year extension, Amendment P00002). No other changes in the project scope or outcomes has been made.

Enrollment numbers for the Veterans with no lifetime history of mTBI (Vet-Cntl) have not been met. We have discussed the current enrollment with our biostatisticians. Our primary analysis, Veterans with mTBI (Vet-TBI) compared to Community Controls (Comm-Cntl) is sufficiently powered to do the proposed analysis. The Vet-Cntl group as available now is sufficient to assess whether deployment effects are a likely confound, and should be pursued. If additional samples become available, we will include them in the analysis.

Changes that had a significant impact on expenditures

We do not anticipate that the regulatory delays will impact expenditures for this project.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report.

6. Products

• Publications, conference papers, and presentations

Relevant Publications

Lusardi, T. A., J. I. Phillips, J. T. Wiedrick, C. A. Harrington, B. Lind, J. A. Lapidus, J. F. Quinn and J. A. Saugstad (2017). "MicroRNAs in Human Cerebrospinal Fluid as Biomarkers for Alzheimer's Disease." J Alzheimers Dis **55**(3): 1223-1233. PMID:27814298

Saugstad, J. A., T. A. Lusardi, K. R. Van Keuren-Jensen, J. I. Phillips, B. Lind, C. A. Harrington, T. J. McFarland, A. L. Courtright, R. A. Reiman, A. S. Yeri, M. Y. S. Kalani, P. D. Adelson, J. Arango, J. P. Nolan, E. Duggan, K. Messer, J. C. Akers, D. R. Galasko, J. F. Quinn, B. S. Carter and F. H. Hochberg (2017). "Analysis of extracellular RNA in cerebrospinal fluid." J Extracell Vesicles **6**(1): 1317577. PMID:28717417

Relevant Presentations

Lusardi, T: MicroRNA in Cerebral Spinal Fluid as Biomarkers of Alzheimer's Disease Risk After Brain Injury. IPR, Feb. 17, 2017

Lusardi, T: MicroRNAs in Human Cerebrospinal Fluid as Biomarkers for Alzheimer's Disease. Extracellular RNA Communication Consortium (ERCC), Nov. 11, 2016

• Website(s) or other Internet site(s)

Nothing to Report.

• Technologies or techniques

Nothing to Report.

• Inventions, patent applications, and/or licenses

Nothing to Report.

• Other Products

Nothing to Report.

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

Name:	**Dr. Joseph Quinn**
Project Role:	PI
Researcher Identifier (ORCID ID):	0000-0001-7305-2256
Nearest person month worked:	0.6
Contribution to Project:	Dr. Quinn has provided clinical guidance, scientific review, and reporting assistance.
Funding Support:	*See below*

Name:	**Dr. Theresa A. Lusardi**
Project Role:	Co – PI
Researcher Identifier (ORCID ID):	0000-0003-0699-5662
Nearest person month worked:	3
Contribution to Project:	Dr. Lusardi has coordinated sample and metadata transfer with University of Washington collaborators, performed QC evaluations for pilot studies, developed the bioinformatics pipeline, prepared reports.
Funding Support:	*See below*

Name:	**Dr. Julie A. Saugstad**
Project Role:	Key Personnel
Researcher Identifier (ORCID ID):	0000-0002-2996-9611
Nearest person month worked:	1
Contribution to Project:	Dr. Saugstad is an expert molecular biologist, who has provided technical and organizational guidance for the miRNA assays.
Funding Support:	Dr. Saugstad's work for this project was funded by this award

Name:	**Dr. Ursula S. Sandau**
Project Role:	Key Personnel
Researcher Identifier (ORCID ID):	0000-0002-3646-7089
Nearest person month worked:	6
Contribution to Project:	Dr. Sandau is an experienced molecular biologist, who has performed all TLDA assays for this project. She has been active in the development of quality assessment standards and interpretation of the results.
Funding Support:	Dr. Sandau's work for this project was funded by this award

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

There have been no changes to active support for Dr. Joseph Quinn

Changes to funding support for Dr. Theresa Lusardi:

FTE	Funding Source	Changes
0.25	USAMRMC W81XWH-15-1-0318	Unchanged from last year
0.02	NIH UH3TR000903	Updated for FY2018
0.73	Computational Biology Program	Added OHSU Computational Biology effort (non-grant)

What other organizations were involved as partners?

Organization Name	Seattle Institute for Biomedical and Clinical Research (SIBCR)
Location of Organization	1660 S. Columbian Way, MS S-151, Seattle, WA 98108-1532
Partner's Contribution	*Collaboration*: Provide banked CSF samples, corresponding clinical and biomarker data. Analytic support, including

	integration of findings resulting from this project with ongoing multimodal studies of the same participant group. Contribute to preparation of abstracts and manuscripts resulting from the research.

8. Special Reporting Requirements

None.

9. Appendices

References

Agarwal, V., G. W. Bell, J. W. Nam and D. P. Bartel (2015). "Predicting effective microRNA target sites in mammalian mRNAs." Elife **4**.

Betel, D., A. Koppal, P. Agius, C. Sander and C. Leslie (2010). "Comprehensive modeling of microRNA targets predicts functional non-conserved and non-canonical sites." Genome Biol **11**(8): R90.

Kertesz, M., N. Iovino, U. Unnerstall, U. Gaul and E. Segal (2007). "The role of site accessibility in microRNA target recognition." Nat Genet **39**(10): 1278-1284.

Miranda, K. C., T. Huynh, Y. Tay, Y. S. Ang, W. L. Tam, A. M. Thomson, B. Lim and I. Rigoutsos (2006). "A pattern-based method for the identification of MicroRNA binding sites and their corresponding heteroduplexes." Cell **126**(6): 1203-1217.

Oliveira, A. C., L. A. Bovolenta, P. G. Nachtigall, M. E. Herkenhoff, N. Lemke and D. Pinhal (2017). "Combining Results from Distinct MicroRNA Target Prediction Tools Enhances the Performance of Analyses." Front Genet **8**: 59.